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Expression of Tolerogenic HLA-G Confer Worse Outcome in Patients with Chronic Lymphocytic Leukemia

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ABSTRACT

Background: Chronic lymphocytic leukemia (CLL) is the most common hematological malignancy in adults presenting with varied clinical course. There is a higher request for founding known prognostic factors for stratifying CLL patients. HLA-G is a non-classical major histocompatibility complex (MHC) molecule. It can be expressed in membrane bound (m.HLA-G) and soluble forms (s.HLA-G).

Objective: The aim of work was to investigate the expression of membrane form of HLA-G in CLL and correlate findings and a variety of clinical and laboratory variables.

Patients and methods: This prospective study included a total of thirty newly diagnosed B-CLL patients, attending at Hematology Unit, Medical Oncology Department, national Cancer institute, Cairo. This study was conducted between December 2017 to July 2019. Diagnosis of CLL was confirmed by flow cytometric immunophenenotyping using standard lymphoma panel, membrane HLA-G was determined by flow cytometry.

Results: The expression of HLA-G by flowcytometry was negatively correlated with the platelet count ($r = -0.516$, $p = 0.004$) and Hb ($r = -0.479$, $p = 0.007$). The expression of HLA-G was significantly higher in CD38 positive cells ($p = 0.006$). The expression of HLA-G was significantly higher in Rai stage 4 compared to stage 1 & stage 2 ($p = 0.001$).

Conclusion: It could be concluded that expression of HLA-G might represent signs of immunosuppression in CLL patients which contribute to the immune escape of tumor cells. In addition, HLA-G expression by B-CLL cells correlates significantly with the known prognostic markers of disease progression, mainly Rai clinical staging, CD38 expression and worse survival, making this parameter possibly an important prognostic factors of disease progression.

Keywords: CLL, HLA-G.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is the most public form of leukemia in the world [(1)]. In Egypt, CLL was the most common subtype of leukemias and reported over 80% of lymphoid leukemias [(2)].

The recorded significant prognostic indicators in CLL are clinical staging systems developed by **Rai *et al.*** [(3)] **and Binet *et al.*** [(4)]. Other prognostic markers include markers of tumor load such as (thymidine kinase and B2-microglobulin), and expression of specific proteins in CLL cells; CD38, CD49d & ZAP-70.

People with CLL have varied clinical course. Some people never need medical treatment however others have more aggressive course demanding early beginning of therapy [(5)].

The well-known Rai and Binet staging systems and the well-known prognostic markers be unsuccessful to recognize B-CLL patients with disease progression who will benefit from early start of therapy and are the target for unique therapy [(6)].

Therefore, increased request for founding known prognostic factors is of great importance. Classical major histocompatibility complex (MHC) class I antigens are very much polymorphic molecules that facilitate the presentation of peptides to T lymphocytes. They are universally expressed and show an significant role in the recognition of tumor cells and their products by the immune system [(7)]. Human leukocyte antigen G (HLA-G) is a non-classical MHC class I antigen with very slight sequence variability which is not expressed in normal tissues except in fetal trophoblasts [(8)].

Both Membrane bound and soluble HLA-G are involved in the development of malignancies in multiple ways [(9)].

HLA-G utilizes various immunoregulatory roles such as inhibition of natural killer (NK) cell or T-cell-mediated cytolysis, induction of T-cell apoptosis, or inhibition of transendothelial NK cell migration [(8)]. Meanwhile the net outcome of these effects is immunosuppression, HLA-G expression in tumor cells could favor their escape from antitumor immune

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responses, consequently permitting tumor progression [9].

HLA-G might be a therapeutic target by blocking HLA-G with specific antibodies or by using HLA-G peptides, inducing the destruction of cancer cells [10]. Ectopic HLA-G expression has been found in various types of malignancies including B-cell and T-cell lymphomas, melanoma, as well as lung, kidney, bladder, breast, malignant ascites, and colorectal carcinoma[9]. Several studies proven that HLA-G expression is associated with poor prognostic markers of a B-CLL , mainly Binet clinical staging and CD38 expression by B-CLL cells, which indicates that this marker may play a role as an important prognosticator of disease progress and therefore may be used as a target therapy in B-CLL [11].

The aims of the current work were to investigate the expression of membrane form of HLA-G in chronic lymphocytic leukemia (CLL), in a trial to evaluate its role as a prognostic marker and tumor escape mechanism and to correlate findings with a variety of clinical and laboratory variables.

PATIENTS AND METHODS

This prospective study included a total of thirty newly diagnosed B-CLL patients, 18 males and 12 females (mean age 58.8 ± 12.1 years), attending at Hematology Unit, Medical Oncology Department, national Cancer institute, Cairo. This study was conducted between December 2017 to July 2019. Written informed consents were obtained from all the participants in the study.

Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee, Faculty of Medicine. Aswan University.

The study included two groups. The first group included 30 newly diagnosed patients with CLL; the CLL Group. The second group included 10 healthy volunteers; the Control Group.

The first group was further divided between two subgroups according to modified Rai staging system **Rai *et al.* [3]; Low risk group** (stage 1 and 2) consisted of (19) patients (63.3%) and **high risk Group** (stage 4) consisted of 11 patients (63.3%)..

Samples were taken at time of diagnosis and patients were analyzed for Biologic and clinical characteristics, including age, sex, presence of lymphadenopathy, organomegaly, rai stage, white blood cell count (using an Advia 60 cell counter; Bayer), Lymphocyte count, hemoglobin concentration, platelet count and serum activities of lactate dehydrogenase, as well as serum concentrations of β 2 -microglobulin, and bone marrow morphological examination, particularly lymphocyte counts.

All patients were receiving the standard treatment combination of fludarabine, cyclophosphamide, and rituximab (FCR), and followed up during course of chemotherapy for 18 months for assessment of overall survival.

For each patient, morphological diagnosis of B-CLL was confirmed by flow cytometry (Beckman-Coulter, Navios), with CLL-typical CD5, CD19, CD23, CD38, CD79b, FMC7 immunophenotypes, surface Ig as well as κ and λ light chain restriction; labelling was performed with fluorescein isothiocyanat (FITC) or phycoerythrin (PE).

CLL was distinguished from other chronic lymphoproliferative disorders according to the scoring system proposed by **Moreau *et al.* [12].**

Blood samples were obtained during routine follow-up visits. Venous blood were withdrawn from each patient and divided into three tubes; the first two tubes contained EDTA for performing complete blood counts (CBC) and peripheral blood films stained with Leishman's stain to determine differential leukocyte counts. The second tube was used for immunophenotyping. The third one for serum biochemistry tests.

Flow-Cytometric Immunophenotyping:

Was done to determine the classical immunophenotyping panel for diagnosis of CLL, it was performed using CLPD panel: using the following panel of monoclonal antibodies was used: (lymphoproliferative diseases panel) CD5, CD19, CD20, CD22, CD23, CD79b, FMC7, CD10, CD38, CD3, CD4,CD8 as well as κ and λ light chains labeled with either fluorescin isothiocyanate (FITC) or phycoerythrin (PE). Samples were considered positive for a marker if ≥ 20% of cells expressed that marker, the whole blood lysis staining method was performed.

- A total of 10,000 events were routinely acquired.
- Anti-CD38 monoclonal antibody (FITC) was used to analyze CD38 expression, and CD38 expression was assessed as the percentage of CD38-positive cells of the gated B cells (CD19+/CD5+).
- Patients were considered positive for CD38 when ≥ 30.0% leukemia cells (CD19+/CD5+) expressed it.

Flow-cytometry immunophenotyping to determine expression of HLA G

- Using a whole-blood lysis method, 100 µl of anticoagulated patient blood were pipetted into a two standard flow-cytometry tubes.
- The tube containing 10 µl of the labeled Anti-HLA G antibody [MEM-G/9] (Phycoerythrin), to analyze HLA-G expression.
- 10,000 events were acquired, Positivity of HLA-G on gated B cells (CD19+/CD5+) were considered positive if ≥ 20% of cells expressed that marker.

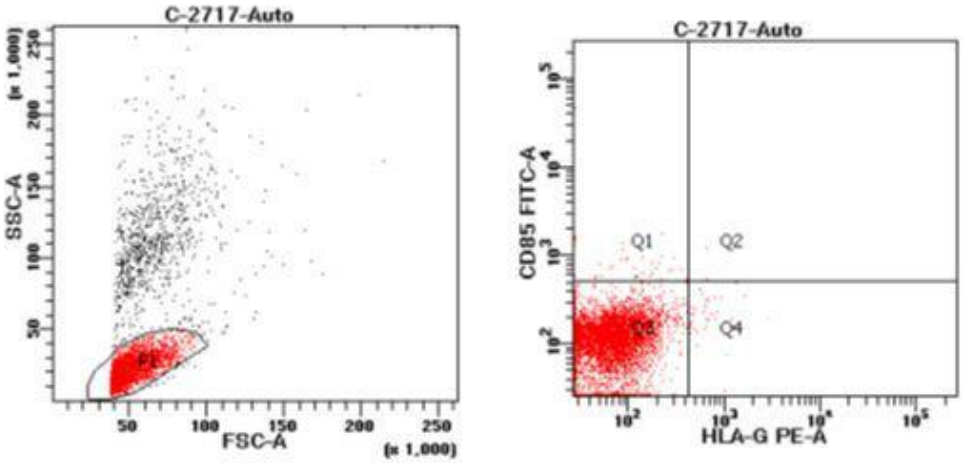

Fig. (1): Dot plot histogram shows HLA G expression on CLL case.

Statistical analysis

Recorded data were analyzed using the statistical package for social sciences, version 20.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were expressed as mean± standard deviation (SD). Qualitative data were expressed as frequency and percentage. Independent-samples t-test of significance was used when comparing between two means. Chi-square (x^2) test of significance was used in order to compare proportions between two qualitative parameters. The confidence interval was set to 95% and the margin of error accepted was set to 5%. The p-value was considered significant as the following: P-value <0.05 was considered significant. P-value <0.001 was considered as highly significant. P-value >0.05 was considered insignificant.

RESULTS

The study included two groups. The first group included 30 newly diagnosed patients with CLL; the CLL Group. The second group included 10 healthy volunteers: the Control Group.

Characteristics of the B-CLL Patients:

To evaluate the prognostic significance of HLA-G in CLL, a cohort of 30 newly diagnosed B-CLL patients [18 males and 12 females (mean age 58.8 ± 12.1 years)].

Table (1): Findings of laboratory tests of the two studied groups

	CLL Group n=30	**Control Group n=10**	**p value**
Hemoglobin (g/dL)	11.0±1.4	12.3±1.5	0.03
TLC ($x10^3/mm^3$)	48.5 ±9.5	5.8 ±1.2	<0.001
ALC ($x10^3/mm^3$)	42.2±6.3	2.2±0.3	<0.001
Platelets ($x10^3/mm^3$)	132 ±15.6	235 ±22.4	<0.001

Data are presented as mean±SD or median (range), TLC: Total leukocytic count, ALC: absolute lymphocyte count.

Table 1: shows that Hemoglobin concentration and platelet count were significantly lower in CLL Group. Meanwhile, total leukocytic count and percentage of lymphocytes were significantly higher in CLL group

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Table (2): Correlation between membrane HLA-G in CLL Group and laboratory parameters (n=30).

		HLA-G membrane-bound
Hemoglobin (g/dL)	r	-0.479
	p	0.007
Platelets ($x10^3/mm^3$)	r	-0.622
	p	0.001

r = correlation coefficient, p = p value

Table (2): shows that the expression of membrane-bound HLA-G was negatively correlated with the platelet count (r = -0.622, p = 0.001) and Hb (r = -0.479, p = 0.007).

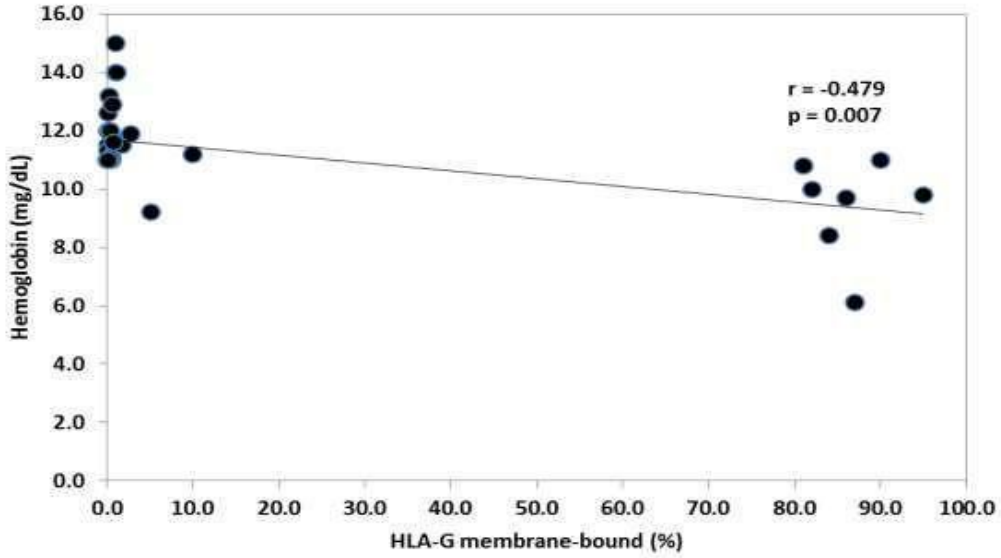

Fig. (2): Correlation between membrane HLA G and Hb.

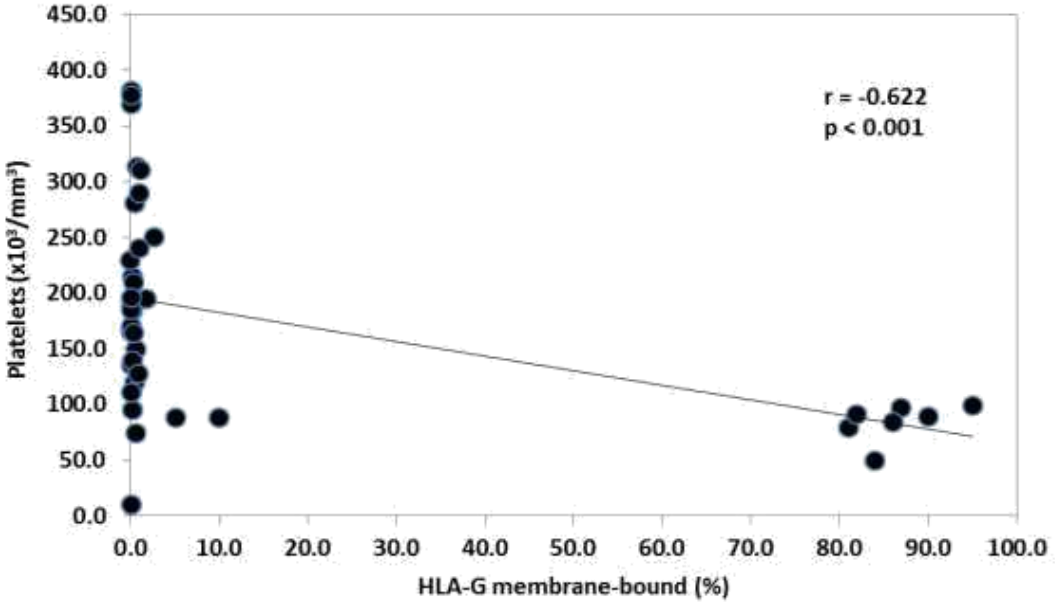

Fig. (3): Correlation between membrane HLA G and platelets.

Table (3): Membrane HLA-G, LDH and β2 microglobulin and in different Rai stages in CLL Group (n=30)

	Stage 1 n=7	**Stage 2 n=12**	**Stage 4 n=11**	**p value**
mHLA-G (%)	0.1 ±0.021	0.2 ±0.43	82 ±18.7	0.001
LDH (U/L)	276.8±91	222.8.1±52.8	281.8.9±92	0.185
β2 mG (µg/ml)	3.5±1.6	3.5±1.0	4.3±1.1	0.064

Table (3): shows that the expression of HLA-G membrane-bound was significantly higher in Rai stage 4 compared to stage 2+1 (p = 0.001). The level of β2 microglobulin was relatively higher in stage 4 compared to stage 1+2, but this difference was not statistically significant (p =0.064).

Table (4): Relations between expression of membrane-bound HLA-G and age, sex, Rai stage

	Expression of membrane-bound HLA-G		**p value**
	≤ 20.0%	**> 20.0%**	
Age			
< 60 years	14 (93.3%)	1 (6.7%)	0.08
≥ 60 years	9 (60%)	6 (40%)	
Gender			
Male	6(30.0%)	4 (70.0%)	0.181
Female	17(60.0%)	3 (40.0%)	
Rai stage			
Stage 1	7 (100.0%)	0 (0.0%)	<0.001
Stage 2	12 (100.0%)	0 (0.0 %)	
Stage 4	4 (36.4%)	7 (63.6%)	

Table (4) shows that higher expression of membrane-bound HLA-G (> 20 %) was significantly more frequent in Rai stage 4 (p<0.001).

Table (5): Relation between expression of membrane-bound HLA-G and laboratory parameters

	Expression of membrane-bound HLA-G		**p value**
	≤ 20%	> 20%	
ALC (x10^3/mm^3)			
≤ 42.2	12(80.0%)	3 (20.0%)	1.000
> 42.2	11 (73.3. %)	4 (26.7%)	
Hemoglobin (g/dL)			
≤ 10	1 (16.7%)	5 (83.3%)	0.001
> 10	22 (91.7%)	2 (8.3%)	
Platelets **(x10^3/mm^3)**			
≤ 150	11 (25.0%)	7 (75.0%)	0.024
> 150	12 (78.6%)	0 (21.4%)	
Lactic acid dehydrogenase (U/L)			
≤ 280	17 (81.0%)	4 (19.0%)	0.640
> 280	6 (66.7%)	3 (33.3%)	
β2 microglobulin (µg/ml)			
≤ 3	10 (90.9%)	1 (9.1%)	0.215
> 3	13 (68.4%)	6 (31.6%)	
CD38 expression			
Positive	6 (42.6%)	7 (53.8%)	0.001
Negative	17 (100.0%)	0 (0.0%)	

Table 5: shows that higher expression of membrane-bound HLA-G (> 20.0 %) was significantly associated with platelet count less than 150 x10^3/mm^3 (p = 0.024), HB < 10 gm /dl (p = 0.001) and positive CD38 expression (p = 0.001). Expression of membrane-bound HLA-G was higher in relation to β2 microglobulin > 3 µg/ml, but the relation was not statistically significance.

Survival Analysis:

The median follow up period was 14.3 months (range: 1.2-21.0 months). At the end of the study 5 cases died. The cumulative overall survival was 83.3%. , Table 6 shows the relation between overall survival and different prognostic factors.

Higher expression of membrane-bound HLA-G was associated with significantly worse survival (p < 0.001) &HB count< 10 gm/dl was associated with significantly worse survival (p <0.001,). Similarly, patients with positive CD38 and those with Rai stage 4 had significantly worse survival (p = 0.005).

Table (6): Overall survival and its relation to the prognostic factors.

	N	No of deaths	Cumulative survival at 18 months (%)	p-value
Whole CLL group	30	5	83.3%	
Membrane-bound HLA-G				
≤ 20.0%	23	1	95.7%	<0.001
> 20.0%	7	4	42.9%	
Hemoglobin (mg/dL)				
≤ 10	6	4	33.3%	<0.001
> 10	24	1	95.8%	
CD38 expression				
Positive	13	5	61.5%	0.005
Negative	17	0	100.0%	
Rai				
Stage 1	7	0	100.0%	0.005
Stage 2	12	0	100.0%	
Stage 4	11	5	54.5%	

DISCUSSION

In our study, 30 newly diagnosed B-CLL patients showed membrane bound HLA-G positivity rate by flow cytometry in the range of (0.10-95.00 %) while control was (0.05-2.70), by using cut off 20.0 %, 7 (23.3 %) patients had HLA G expression above 20.0% (HLAG- positive group) while in the other 23 (76.6 %) patients HLA-G expression in leukemic cells was <20% (HLAG- negative group).

Like **Attia *et al.*** [13] we involved direct immunofluorescence using the MEM/ G9 antibody. This may be a more sensitive approach to visualize the HLA-G antigen. Furthermore, we measured HLA-G expression in CD19+/CD5+ leukemic cells.

In the present work, there was a significant difference between the two groups of patients regarding Hb concentration and platelet count. They were significantly lower in the HLA-G-positive group compared with the HLA-G-negative one (p = 0.001, 0.024 respectively).

These findings are in accordance with previous studies by **Amiot *et al.*** [14]**; Rebmann *et al.*** [15] **and Erikci *et al.*** [16]**.** In contrast, **Nückel *et al.*** [17] found no significant difference between both groups regarding platelet or HB. However **Attia *et al.*** [13] found significant correlation regarding platelets count but not Hb.

The best commonly used prognostic factors in patients with B-CLL is the staging system developed by Rai [18]**.**

In our study, a statistically significant association was found between advanced stages of the disease (Rai stages 4), which had the poorest prognosis, and the higher expression of HLA-G by leukemic cells (p <0.001).

These results are reliable with previous studies by **Erikci *et al.*** [16] **and Attia *et al.*** [13] whom establish important correlation with binet stage (B and C) in contrast.

Also, **Ozet *et al.*** [11] stage 3 and 4 with HLA-G, found significant correlation between rai while **Nückel *et al.*** [17] found no HLA-G Positive and -negative groups significant difference between regarding Binet stages. CD38 is a type II membrane glycoprotein whose expression was varied among CLL patients [19].

Important prognostic changes were found between CD38-positive and -negative groups of patients concerning chemotherapy requirements and overall survival [20]**.**

The interactions between CD38 and its ligand CD31, discharge -apoptotic signals affecting the leukemic cells which lead to increase leukemic cells overgrowth and poor prognosis [21]**.**

In this study, there was a strong association between higher expression of HLA-G and CD38 expression by leukemic cells (p = 0.001). This finding was in agreement with previous study by **Attia *et al.*** [13] **and Ozet *et al.*** [11]**.**

While, **Giannopoulos *et al.*** [22] found no correlation between HLA-G expression and the known prognostic markers of B-CLL (ZAP-70 and CD38).

Nückel *et al.* [17] also did not find any correlation between HLA-G and ZAP-70 or CD38, but he found that the higher HLA-G levels give rise a significantly shorter time to progression, proposing that HLA-G might help as an important prognostic factor for B-CLL patients.

We found that Higher expression of membrane-bound HLA-G was associated with significantly worse survival (p < 0.001) & Similarly, patients with positive CD38 and those with Rai stage 4 had significantly worse survival (p = 0.005).

CONCLUSION

It could be concluded that expression of HLAG might represent hallmarks of immunosuppression in CLL patients which contribute to the immune escape of

tumor cells. In addition, the present study demonstrated that HLA-G expression by flow cytometry on B-CLL cells correlates significantly with the known prognostic markers of disease progression, mainly Rai clinical staging, CD38 expression and worse survival, which make these parameters a possibly important prognosticators of disease progression.

Although several laboratory tests such as IGHV mutation and cytogenetic investigations are not performed in many laboratories due to their high cost, flow cytometric methods remain relatively cheaper and easier.

Once the optimum cut-off level and standardized method for HLA-G expression by flow cytometry are determined, it could be used as a prognostic factor.

RECOMMENDATIONS

Based on the previously mentioned data, we recommend the followings:

1- Large scale studies are recommended in a larger number of cases and for long-term follow-up.
2- HLA G measured by flow cytometry is a powerful prognostic parameter in patients with CLL and should be included in the routine immunophenotyping pannel of CLL.
3- The optimal cut-off points of HLA G to stratify patient risk will need to be defined in future studies utilizing even larger cohorts.
4- Further studies must be including other prognostic indicators in CLL patients such as: immunoglobulin heavy-chain gene rearrangement (IGHV) mutation status and other cytogenetic abnormalities such as: del 11q22-23, del 17p and del 13q 14.3 should be done.
5- Possible use of HLA-G as a target for therapy of B-CLL are required.

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